Supporting Information

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SI Materials and Methods

Electrophysiology and Pharmacology. Patch-clamp measurements were performed on acutely dissected adult fly brains as described previously (1, 2). l-LNv recordings were made in whole-cell current-clamp mode. After allowing membrane properties to stabilize after whole-cell break-in, 30–60 s of recording in current-clamp configuration (unless otherwise stated) was obtained under dark conditions (~0.05 mW/cm^2) before lights were turned on. For most experiments, unless stated otherwise, lights-on data were then collected for 30–60 s and followed by another 30–60 s of darkness. DPI and H_2O_2 were obtained from Sigma and stock solutions prepared in double distilled H_2O or standard external recording solution and bath-perfused onto the brain preparation in standard recording solution (1).

Optics. Multiple light sources were used for these studies (1, 2). The standard halogen light source on the Olympus BX51 WI microscope (Olympus) was used for all experiments with white light. Wavelength isolation of 375–450 nm, <550 nm, and >550 nm for electrophysiological recordings was achieved by placing appropriate combinations of 25-mm long- and short-pass filters (Edmund Industrial Optics) over the halogen light source directly beneath the recording chamber. Filters were changed during recordings to internally match neuronal responses to varying wavelength ranges. Some recordings using intense blue light (450–490 nm, 19 mW/cm^2) were obtained by using the standard mercury light source fitted to the Olympus BX51 WI microscope with cut-on and cutoff wavelengths determined by the standard GFP filter cube. In most cases, the entire matrix of white and isolated wavelengths was obtained from each single recording, with at least two light/dark cycles per wavelength range per recording. For the behavioral experiments, we used ultra high-power LEDs implemented by Stanford Photonics, with the UHP-Mic-LED-460, which provides >1 W collimated blue light (460 nm peak, 27 nm spectrum half width, 85% peak power at 450 nm) or the UHP-Mic-LED-595, which provides >650 mW collimated orange light (595 nm peak, 16 nm spectrum half width). The LED devices were triggered on and off manually for behavioral experiments. Light was measured for all sources by using a quantum sensor and a light meter (LI-250A; LI-COR) and expressed as mW/cm^2.

Light Response Behavioral Testing. The automated TriKinetics *Drosophila* Activity Monitor system was used to assay the locomotor activity of individual flies (3, 4). Adult male flies (1–4 d old) were placed singly in glass tubes, and their locomotor activity was tracked by the breaking of an IR beam recorded on a data acquisition computer. Behavioral activity counts were collected throughout the experiment in 1-min bins. For all assays flies were entrained to at least 3 d of standard 12-h:12-h light: dark cycles (light phase illumination was 50–100 mW/cm^2). LED light pulses (5-min duration, blue 460 nm or orange 595 nm) were given at three hourly intervals starting at 6 h after lights off for three nights in a row. For data analysis, for each pulse, the locomotor activity of individual flies are summed during the 5-min light pulse, this sum is divided by the locomotor activity of the same individual for 5 min immediately preceding the light pulse, then these values are averaged across each genotype and expressed as the mean and SEM of beam crossings per pulse. ANOVA is used to determine whether there are differences between genotypes.

Genetics. The control genotype *w; pdfGAL4-dORK-NC1-GFP* was generated by recombination between the driver and channel-GFP fusion lines. "Rescue" experiments were performed with genotypes *w* or *hk*$^{-/-}$ *;pdfGAL4-dORK-NC1-GFP/UAS-(dCRY, Hk-WT, Hk-D260N, Hk-K289M*); *cry01* (homozygous) or + as described in Dataset S1.

Cell Culture and Transfection. HEK 293 human embryonic kidney cells were maintained as described previously (5, 6). Cells were cultured in MEM with L-glutamine and Earle salts supplemented with 10% FBS. Each 60-mm dish of ~80% confluent cells was transfected as previously described (6). All cDNA constructs for transfection of HEK 293 cells were under the control of the mammalian cytomegalovirus CMV promoter using the the pCS2+ vector (7).

Statistics. Data are presented as mean ± SEM. Values of *n* refer to number of measured lights on/off cycles; in all cases, the *n* values were obtained from at least five separate recordings. All statistical tests of ANOVA were performed with SigmaPlot 11. Variables were first tested for normality; if found to fail normality testing, Kruskal–Wallis one-way ANOVA on ranks was performed, followed by Dunn test. ANOVA on normally distributed variables was followed by Tukey test to determine significant differences between genotypes. For before and after lights-on comparisons of two sets of normally distributed variables, paired *t* tests were performed with SigmaPlot; for non-normally distributed variables, values were compared by using a signed-rank test (SigmaPlot).

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2. Fogle KJ, Parson KG, Dahm NA, Holmes TC (2011) CRYPTOCHROME is a blue-light sensor that regulates neuronal firing rate. *Science* 331(6023):1409–1413.
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4. Sheeba V, et al. (2008) Large ventral lateral neurons modulate arousal and sleep in Drosophila. *Curr Biol* 18(20):1537–1545.
5. Holmes TC, Fadool DA, Ren R, Levitan IB (1996) Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. *Science* 274(5295):2089–2091.
6. Fadool DA, Holmes TC, Berman K, Dagan D, Levitan IB (1997) Tyrosine phosphorylation modulates current amplitude and kinetics of a neuronal voltage-gated potassium channel. *J Neurophysiol* 78(3):1563–1573.
7. Nitabach MN, et al. (2001) A mechanism for combinatorial regulation of electrical activity: Potassium channel subunits capable of functioning as Src homology 3-dependent adaptors. *Proc Natl Acad Sci USA* 98(2):705–710.

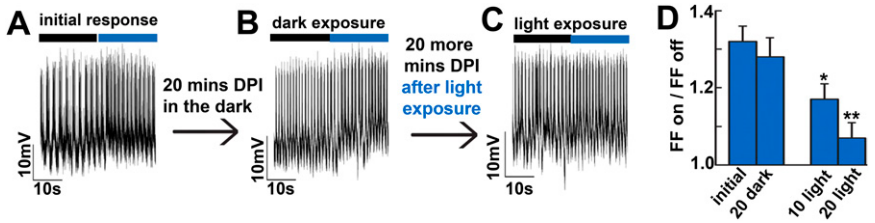

Fig. S1. The l-LNv light response requires a CRY-specific FAD redox reaction. (*A*) Representative recording of control l-LNv blue light response (black bar indicates no light, purple bar indicates 375–450 nm blue light, 0.6 mW/cm^2). (*B*) Representative intact l-LNv light response (firing frequency lights on/lights off) following 16.8 μM DPI perfusion in the dark for 20 min. (*C*) At 15 min after first light exposure (35 min total DPI), the light response is reduced. (*D*) Bar graphs quantifying the l-LNv response to blue light. The initial blue light response following 20 min of DPI (1.28 ± 0.05, $n = 10$) does not differ from non-DPI controls (1.32 ± 0.04, $n = 39$, $P = 0.93$). After 10–15 min DPI following initial exposure to light (35 min DPI exposure), the blue light response is lower (1.17 ± 0.04, $n = 9$, $P = 0.15$ vs. non-DPI control recordings). By 20–25 min after light exposure (40–45 min DPI exposure), the blue light response is significantly lower (1.07 ± 0.04, $n = 7$, $P = 0.011$ vs. controls).

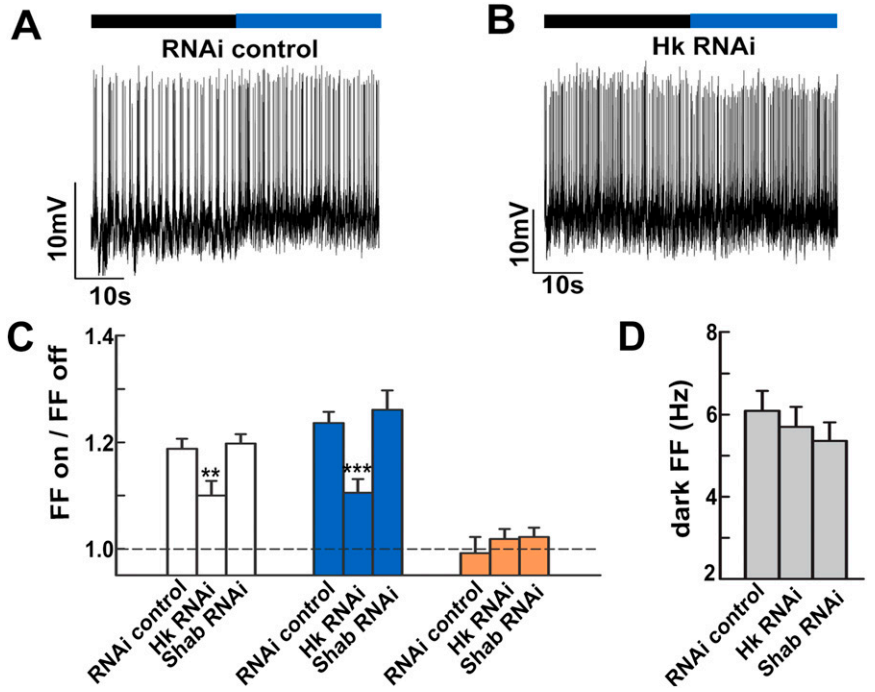

Fig. S2. LNv-directed expression of *hyperkinetic* RNAi significantly knocks down the l-LNv light response. (*A*) Representative traces for the intact control blue l-LNv light response (0.6 mW/cm^2, 375–450 nm, blue bar; black bar indicates no light) recorded from RNAi control genotype flies (*w, pdfGAL4;pdfGAL4-UAS-DCR/p12c*) vs. (*B*) Significant attenuation of the blue light response recorded from the l-LNv of flies that express Hk RNAi and DICER in the LNv (*w, pdfGAL4; pdfGAL4-UAS-dcr/p12c;UAS-hk-RNAi*). (*C*) Bar graph quantifies the l-LNv white (white bars, 4 mW/cm^2 unfiltered halogen), blue (blue bars, 0.6 mW/cm^2, 375–450 nm blue), and orange (orange bars, 4 mW/cm^2, >550 nm orange) light response for RNAi control, Hk RNAi, and Shab RNAi flies. The l-LNv white light response of control flies (1.19 ± 0.02, $n = 16$) is significantly different from Hk RNAi (1.10 ± 0.03, $n = 28$, $P = 0.03$) but not control Shab RNAi flies (1.20 ± 0.02, $n = 17$, $P = 0.96$). Similarly, the l-LNv blue light response of control flies (1.23 ± 0.02, $n = 18$) is significantly different from Hk RNAi (1.10 ± 0.03, $n = 31$, $P = 0.006$) but not control Shab RNAi flies (1.26 ± 0.04, $n = 20$, $P = 0.78$). The l-LNv orange light responses do not differ between the RNAi control (0.99 ± 0.03, $n = 15$), Hk RNAi (1.02 ± 0.02, $n = 20$, $P = 0.79$), and Shab RNAi (1.02 ± 0.02, $n = 13$, $P = 0.84$) genotypes. (*D*) The basal dark firing frequencies for RNAi control flies (6.09 Hz ± 0.49, $n = 27$), Hk RNAi flies (5.70 Hz ± 0.49, $n = 37$), and Shab RNAi flies (5.36 Hz ± 0.45, $n = 29$) do not differ ($P = 0.96$ and $P = 0.80$ vs. control, respectively). All data values and statistics are presented in detail in Dataset S1.

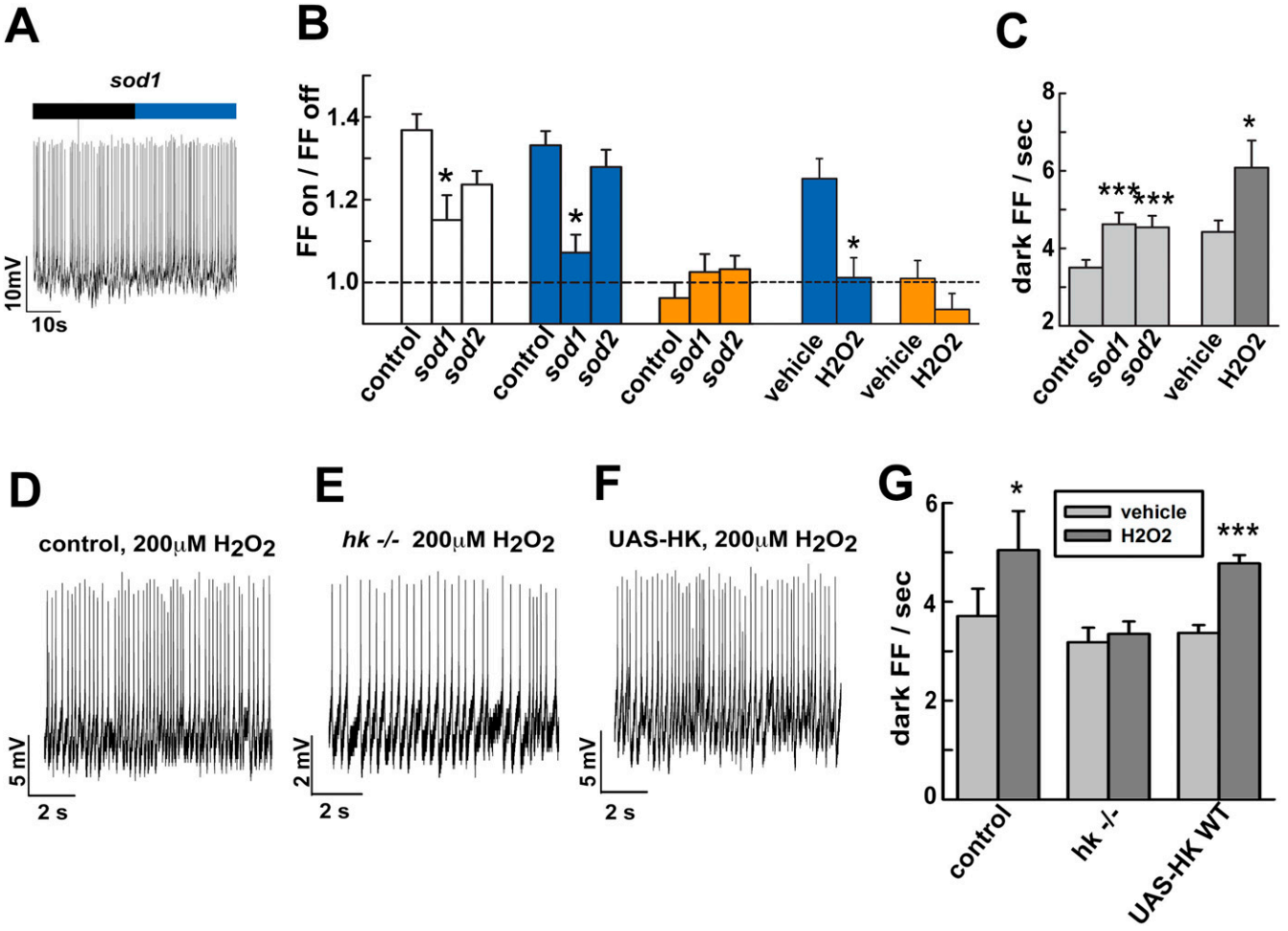

Fig. S3. The l-LNv light response is occluded by genetic or chemical disruption of the cellular redox environment in an Hk redox sensor-dependent manner. (*A*) Representative recording of *sod1*$^{-/-}$ (*sod1*) l-LNv. (*B*) The l-LNv response of *sod1*$^{-/-}$ (1.15 ± 0.06, $n = 11$) to white light (4 mW/cm^2) is significantly lower than control (1.36 ± 0.04, $P = 0.023$, $n = 13$), whereas that of *sod2*$^{-/-}$ (*sod2*) (1.23 ± 0.03, $n = 14$) does not differ from control ($P = 0.14$). *sod1*$^{-/-}$ also exhibits significantly attenuated l-LNv blue light (0.6 mW/cm^2, 375–450 nm) response (1.07 ± 0.04, $n = 8$) relative to control (1.33 ± 0.03, $n = 16$, $P = 0.01$), whereas *sod2*$^{-/-}$ mutants do not differ from control (1.27 ± 0.04, $n = 14$, $P = 0.53$). Control (0.96 ± 0.04, $n = 16$), *sod1*$^{-/-}$ (1.025 ± 0.04, $n = 8$), and *sod2*$^{-/-}$ (1.03 ± 0.03, $n = 14$) all show no significant response to orange light (4 mW/cm^2, >550 nm). Acute treatment with H_2O_2 (200 μM) significantly abolishes the l-LNv response to blue light (1.01 ± 0.05, $n = 11$, $P = 0.0007$, 375–450 nm blue light, 0.6 mW/cm^2) relative to control (1.25 ± 0.05, $n = 8$), whereas there is no significant response to orange light (4 mW/cm^2, >550 nm) in vehicle control (1.01 ± 0.04, $n = 7$) or H_2O_2 conditions (0.93 ± 0.04, $n = 11$). (*C*) Dark spontaneous firing frequency is significantly greater ($P < 0.01$) in *sod1*$^{-/-}$ (4.62 Hz ± 0.3, $n = 41$, $P = 0.001$) and *sod2*$^{-/-}$ (4.54 Hz ± 0.3, $n = 61$, $P < 0.001$) relative to control (3.50 Hz ± 0.2, $n = 49$). Dark spontaneous firing frequency is significantly greater following H_2O_2 treatment (6.08 Hz ± 0.7, $n = 21$) relative to vehicle control (4.42 Hz ± 0.3, $n = 23$, $P = 0.03$). (*D*) Representative recording of l-LNv firing in dark in 200 μM H_2O_2 treatment for WT genetic control vs. (*E*) genetic null *hk*$^{-/-}$, (*F*) and WT Hk expressed in LNv in *hk*$^{-/-}$ genetic background (Hk WT rescue). (*G*) Dark spontaneous firing frequency in l-LNv is significantly higher following H_2O_2 treatment (5.04 ± 1.58, $n = 5$) compared with vehicle treatment (3.71 ± 1.10, $n = 5$, $P = 0.020$) in neurons from WT control flies. Dark spontaneous firing frequency in l-LNv following H_2O_2 treatment in genetic null *hk*$^{-/-}$ (3.35 ± 0.60, $n = 7$) does not differ from vehicle control treatment (3.18 ± 0.71, $n = 7$, $P = 0.249$). Expression of WT Hk in the LNv of genetic null *hk*$^{-/-}$ shows significant increase in l-LNv dark spontaneous firing frequency following H_2O_2 treatment (4.77 ± 0.37, $n = 6$) compared with vehicle treatment (3.37 ± 0.35, $n = 6$, $P \leq 0.001$), showing functional rescue and Hk dependence for the H_2O_2 induced increase in dark spontaneous firing rate. l-LNv Firing rates for vehicle controls for genetic WT control flies, genetic null *hk*$^{-/-}$ flies, and Hk WT rescue flies do not differ. All data values and statistics are presented in detail in Dataset S1.

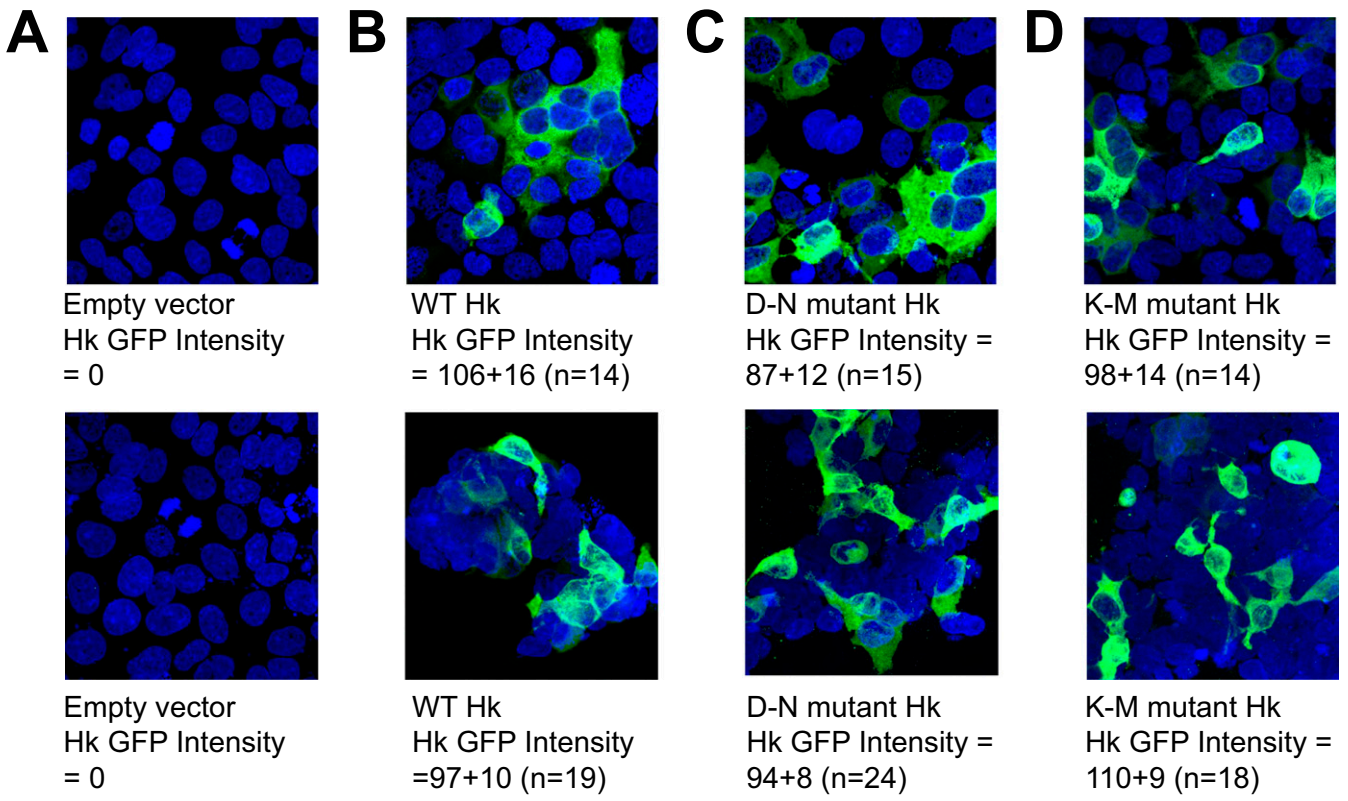

Fig. S4. Hk mutants express at equivalent levels to WT Hk. Expression of *Drosophila* Hk WT GFP, Hk D260N GFP, and Hk K289M GFP (*A*–*D*) in HEK293a grown in DMEM media. Cells (2×10^5) were plated in each well in a 12-well plate maintained overnight at 37 °C. HEK293a cells were transfected with 1.2 μg empty pCS2+ vector (*A*), Hk WT GFP-pCS2+ (*B*), Hk D260N GFP pCS2+ (*C*), or Hk K289M GFP pCS2+ (*D*). At 48 h later, cells were fixed, immunostained with anti-EGFP (green), and counterstained with DAPI (blue). The images were scanned with a Zeiss LSM700 confocal microscope by using Zen software. (*A*–*D*) *Z*-stack image ranges from 16 to 26 μm in thickness. Cell images were analyzed with Volocity 6.3 software (Perkin-Elmer). Hk mutant expression normalized to DAPI express at equivalent levels compared with WT Hk. Fluorescence values are expressed as pixel intensity per cubic millimeter. There are no significant differences between WT and mutant Hk expression (one-way ANOVA). Results of two of six representative experiments are shown in the upper and lower panels.

Other Supporting Information Files

Dataset S1 (XLS)